African Journal of Biotechnology Vol. 4 (5), pp. 449-459, May 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

Full Length Research Paper

Application of biotechnology in fish breeding. II: production of highly immune genetically modified redbelly tilapia, *Tilapia zillii*

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Accepted 18 February, 2005

The present study aims to produce a highly immune, genetically modified redbelly tilapia, *Tilapia zillii* with accelerated growth as a result of direct injection of shark (*Squalus acanthias* L.) DNA into skeletal muscles of fish fingerlings at concentrations of 10, 20, 40 and 80 µg/fish. The results showed that the fish injected with 40 µg/fish had significant (P≤0.05) superiority of growth performance. Also, the body composition of these fish was improved. The fish injected with 20 and 40 µg/fish had significant (P≤0.05) higher means of total antibody activity (total IgM), serum total protein and globulin compared with other injected groups. Cytological examination for all injected fish and their control revealed the same diploid number (2n=44) of chromosomes, and no chromosomal abberations were detected. Moreover, DNA fingerprinting showed high polymorphism among injected fish. Therefore, variable fragments of shark DNA may randomly integrated into *T. zillii* muscle genomes. The present investigation revealed also that egg diameters of injected females and their control were divided into nine groups, which varied between 0.2 and 1.7 mm. In addition, ovary of *T. zillii* females injected with 10; 20; 40 and 80 µg/fish of shark DNA showed 10, 38, 65 and 18% normal oocytes, respectively. Also, test of males injected with various amounts of DNA showed large number of abnormalities. Moreover, the comparison between all injected fish revealed that the testes and ovaries of fish injected with 80 µg/fish were more deformed and atretic. This means that the effects of intramuscular direct injection of foreign DNA into *T. zillii* could be limited to germ cells of fish. Therefore, further studies about the establishment of these effects on the following generations are needed. The result indicates a possible easy and rapid way for improving fish characteristics.

Key words: Genetically modified, highly immune, accelerated growth, *Tilapia zillii*, shark DNA, molecular alterations, chromosome investigations, oocyte diameter, gonad histological changes.

INTRODUCTION

The development of genetically modified fish has undergone intensive research since the first production of genetically modified mammels (Devlin et al., 1995). Genetically modified fish are being developed for both academic and applied goals, allowing the production of useful model systems as well as new genetic strains with improved characteristics for aquaculture (Maclean and Penman, 1990; Chen and Powers, 1990; Houdebine and Chourrout, 1991; Fletcher and Davies 1991; Maclean and Laight 2000). A variety of genes have now been introduced into fish with the goal of influencing traits such as growth, maturation, freezing tolerance, flesh quality and disease resistance (Shears et al., 1991; Chatakondi et al., 1995; El-Zaeem, 2001, 2004; Dunham et al., 2002; El-Zaeem and Assem, 2004).

A foreign gene can be transferred into fish *in vivo* by introducing DNA either into embryos or directly into somatic tissues of adults (Sudha et al., 2001). Direct delivery of DNA into fish tissues is a simple approach,

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providing fast results and eliminating the need for screening transgenic individuals and selecting germline carriers. Gene transfer and expression following intramuscular direct injection of foreign DNA into skeletal muscles of fish has been achieved by several studies (Hansen et al., 1991; Rahman and Maclean, 1992; Anderson et al., 1996; Tan and Chan, 1997; Xu et al., 1999; El-Zaeem, 2004; El-Zaeem and Assem 2004; Hemeida et al., 2004). Moreover, Sudha et al., (2001) stated that the expression of muscular injection of DNA was evident in several non muscle tissues, such as skin epithelia, pigment cells, blood vessel cells and neuron-like cells.

Immunological or physiological parameters from healthy fish may be obtained from blood samples as indirect measurments of disease resistance. One of these parameters could be total antibody activity (total IgM) (Fjalested et al., 1993). The levels of immunoglobulin (IgM) and serum proteins that are present in the blood serum indicate the progress of immune system development. Immunoglobulins are special proteins found in the membrane of lymphocytes that circulate in blood and act as antibodies. IgM is one of the immunoglobulins that is responsible for part of the immune response to bacterial invasion (Magnadottir, 1990 and 1998; Magnadottir and Guomundsdottir, 1992). Marchalonis et al. (1993) reported that IgM may comprise as much as 50% of serum proteins in the shark, while in humans it comprises less than 5%. Magnadottir (1998) isolated IgM from four teleost species; Atlantic salmon (*Salmo salar* L.), halibut (*Hippoglossus hippoglossus* L.), haddock (*Melanogrammus aeglefinus* L.) and cod (*Gadus morhua* L.), with concentrations of 2, 8, 13 and 20% of the serum proteins, respectively.

We have previously reported (El-Zaeem and Assem, 2004) that a hyperimmune genetically modified Nile tilapia, *Oreochromis niloticus* with accelerated growth and improved of body composition can be produced by direct injection of liver shark DNA into skeletal muscles of fish.

Therefore, the aim of this work was to study the effect of direct injection of shark (*Squalus acanthias* L.) DNA into skeletal muscles of redbelly tilapia, *Tilapia zillii*, on growth performance, body composition, immunological and biochemical characteristics of blood serum, oocyte diameters and gonad histological characteristics. Moreover, cytological investigations and DNA fingerprinting of normal and modified fish were carried out.

MATERIALS AND METHODS

Fish origin

The *T. zillii* used in this study descended from a randomly mating population at the laboratory of Breeding and Production of fish, Animal and fish production Department, Faculty of Agriculture, (Saba-Bacha) Alexandria University, Alexandria, Egypt.

Preparation of genomic DNA

High molecular weight DNA was isolated from liver sample of shark according to Brem et al. (1988) method. Isolation of DNA was accomplished by reducing liver sample to small pieces, which were then transferred to a microfuge tube and incubated to overnight until the sample was digested in a buffer containing 50 mM Tris, 100 mM EDTA (pH 8.0), 100 mM Nacl, 0.1 % SDS and 0.5 mg/ml proteinase K. After incubation, samples were extracted twice for 15-20 min with one volume of phenol/chloroform (1:1) and then again twice for 15 min with one volume of chloroform/isoamyle-alcohol (24:1). The aqueous phase was then precipitated with 2.5 volumes of 100% ethanol in the presence of 1/10 volume 3 M sodium acetate (pH 6.0). The pelleted DNA was washed with 70% ethanol and dissolved in 0.1x SSC buffer (saline sodium citrate). The DNA concentrations were measured by UV spectrophotometry. The extracted DNA was restricted by EcoR1.

Culture conditions

Fifty fingerlings of *T. zillii* with an initial live weight and total length (5.25±1.00 g and 7.12±0.50 cm, respectively) were divided randomly into five groups and tagged individually by transbody tags method (Nielsen, 1992), using Tag-gun. Each group was stocked separately at a rate of 1.0 fish/17.5L in a half of rectangle fiberglass tank (total volume, 350 L), which was divided by plastic sieved connected with iron frame. Each tank was supplied with fresh water at a rate of 0.5 L/min with supplemental aeration. Fish were fed twice daily with pellet diet (30% protein), to satiation, six days a week, and weighed biweekly for 90 days.

Injection of foreign DNA *in vivo*

Using 0.1x SSC buffer, four concentrations, 10, 20, 40 and 80 µg/0.1ml/fish, of shark DNA were prepared and injected into skeletal muscles of *T. zillii in vivo* using a hypodermic needle. The fifth group of fish was left without injection as control.

Quantitative traits measurement

The following parameters were measured; body weight, weight gain, daily gain (DG, g/day), specific growth rate (SGR, %/day), total body length, and condition factor (K). Whole body composition of injected fish and their control were analyzed according the standard methods (AOAC, 1984) for moisture (oven drying), protein (micro-kjeldahl method) and lipid (ether extract method).

Immunological and biochemical characteristics

By the end of experiment, blood samples were taken from caudal vein of fish. Serum was obtained by centrifugation of blood at 3000 rpm for 20 minutes and stored at –20°C for later analysis. Serum total protein was measured by Biuret method, as described by Armstrong and Carr (1964). Albumin concentration was determined according to the method of Doumas et al. (1971), using commercial kits (Diamond Diagnostics®). Globulin concentration was estimated by subtraction of albumin concentration from serum total protein value. Finally the purification and concentration of IgM antibodies was measured according to Ohta et al. (1990); Nevens et al. (1992) and Wendelborn et al. (1992), using commercial kit (Immunopure® IgM purification kit).

Chromosome preparations

Chromosomes were preparaed according to (Chourrout and Happe, 1986) method. By the end of experiment, the modified fish and their control were injected into the dorsal muscle with 0.5% colchicine (0.3 ml/100 g in 0.8% Nacl) and kept in well aerated aquarium. After four hours, the fish were sacrificed, anterior kidney was transferred into 2ml of a hypotonic medium (0.8% trisodium citrate) and rapidly dissociated by repeated pipetting. The 5 ml tube was then filled to the top, closed and shaken periodically for 20 min at 20°C. After cetrifugation (100 g for 7 min), the medium was replaced by freshly prepared ethanol-acetic acid (3:1) at 4°C, the first fixation lasting 30 min. The latter operation was repeated twice and the tubes were kept overnight at 4°C; 2ml of fresh fixative were used to resuspend the cells prior to slide preparation. Five drops of the cell suspention were dropped onto a cool slide (covered by a thin layer of deionized water) which was heated twice (once briefly and the other longer), air dried and finally stained in 4% Giemsa for 30 min.

Random amplified polymorphic DNA (RAPD) analysis

By the end of experiment, genomic DNA was extracted from muscle tissues of injected fish and their control according the method described by Brem et al. (1988). The polymerase chain reaction mixture (25 µl) consisted of 0.8 U of Taq DNA polymerase, 25 pmol of dNTPs, 25 pmol of random primer, 2.5 ul of 10X Taq DNA polymerase buffer and 40 ng of genomic DNA. The random primer sequences used in this study were (5′-ACCGGGAACG-3′ and 5′-ATGACCTTGA-3′). The final reaction mixture was placed in a DNA thermocycler. The PCR programme included an initial denaturation step at 94°C for 2 min followed by 45 cycles with 94°C for 30 s for DNA denaturation, annealing at 46°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 10 min were carried out. The samples were cooled at 4°C. Amplification products were electrophoretically separated on 2.5% agarose gel and stained with ethidium bromide, visualized with ultaviolet light and photographed. DNA fragment lengths were determined by comparisons with 100 pb DNA marker (1500, 1000, 500, 300, 100 pb).

Egg diameter and histological characteristics of gonads

By the end of experiment, gonads were carefully removed and weighed then fixed in 10% formol saline solution. Pieces of fixed ovary were examined under binuclear microscope to determine the oocyte diameters. The oocyte diameters were divided into nine groups, the first three groups (0.2, 0.4 and 0.6 mm) are small and transparent, while the remaining ova ranging between 0.8 mm and 1.7 mm in diameter are yolky. For histological study the fixed gonads were washed in 70% ethyl alcohol for three days prior to dehydration, then cleared and embedded in paraffin wax. Sections of 6-10 µm thick were stained with Eirlich hematoxylin and eosin (H&E).

Statistical analysis

Data of the quantitative traits were analyzed using the following model (CoStat, 1986):

$$Y_{ij} = \mu + T_i + B_j + E_{ij}$$

Where Y_{ij} is observation of the ij^{th} parameter measured; µ, overall mean; T_i, effect of i^{th} dose; B_j, effect of J^{th} block; and E_{ij}, random error. Significant differences (P≤0.05) among means were tested by Duncan´s multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

Productive traits

The present study showed a significant effect of different doses of shark DNA injected into *T. zillii* on growth performance and body composition. At the start of experiment, insignificant differences (P≤0.05) were detected in initial body weight and length (IBW and IBL) of *T. zillii*. The highest mean of FBW, WG and DG were achieved by the fish injected with 40 µg of DNA, showing significant improvement (P≤0.05) compared with the other injected and control groups. Also, the highest record of SGR (%/day) was achieved by the fish injected with 40 µg of DNA which was significantly (P≤0.05) differed than the control, but did not differ significantly from that of fish injected with the other doses of shark DNA. The highest condition factor (K) was recorded by the fish injected with 40 and 80 µg of DNA which was significantly (P≤0.05) higher than that of the control and the other injected groups. Final body length (FBL) was significantly (P≤0.05) increased by the fish injected with 40µg of DNA, showing higher mean, compared with the fish injected with 80 µg of DNA and control groups, but did not differ significantly from that of fish injected with 10 and 20 µg of shark DNA (Table 1). The results of growth performance manifested that the dose of 40 µg of shark DNA was more effective in stimulating these traits. These results are consistent with those obtained previously (El-Zaeem, 2004; El-Zaeem and Assem, 2004), which indicate that the optimal dose of foreign DNA injected into fish muscles is 40 µg/fish. Low doses of foreign DNA have been shown by several investigators to be effective in stimulating growth performance and body composition (El-Fiky and Mehana, 1998; El-Zaeem, 2001).

The results of body composition showed that the moisture content was significantly decreased (P≤0.05) by the fish injected with 40 µg of shark DNA, while crude protein was significantly increased (P≤0.05) by the fish injected with 40 µg of shark DNA. Moreover, the highest records of crude fat was obtained by the fish injected with 40 µg of DNA which was significantly (P≤0.05) higher than that of the fish injected with 10 µg of DNA and control groups, but did not differ significantly from that of the fish injected with 20 and 80 µg of shark DNA (Table 1). These results were in agreement with our previous findings (El-Zaeem, 2004; El-Zaeem and Assem, 2004). Also, Chatakondi et al. (1995) and Dunham et al. (2002) reported that the moisture content was lower while the protein content was higher in transgenic common carp muscles containing rainbow trout growth hormone gene compared with their control. Moreover, Martinez et al. (2002) and Lu et al. (2002) observed that anabolic stimulation and average protein synthesis were higher in transgenic than that of non-transgenic fish.

Table 1. Effect of different doses of Shark DNA injected into skeletal muscles of *T. zillii* on growth performance and body composition[1].

Quantitative traits	Doses of shark DNA				
	Control	10 µg	20µg	40µg	80µg
At the start:					
IBW (g)	4.97	5.13	5.27	5.60	5.27
IBL (cm)	7.03	7.07	7.27	7.20	7.03
At the end:					
FBW (g)	11.07^{c}	15.07^{b}	15.37^{b}	18.87^{a}	14.80^{b}
WG (g)	6.10^{c}	9.93^{b}	10.10^{b}	13.27^{a}	9.53^{b}
DG (g/day)	0.05^{c}	0.08^{b}	0.08^{b}	0.11^{a}	0.08^{b}
SGR %/day	0.67^{b}	0.93^{ab}	0.90^{ab}	1.02^{a}	0.88^{ab}
FBL (cm)	8.67^{c}	9.43^{ab}	9.50^{ab}	9.80^{a}	9.03^{bc}
K	1.70^{b}	1.80^{b}	1.79^{b}	2.00^{a}	2.00^{a}
Moisture (%)	75.01^{a}	74.23^{ab}	71.80^{c}	70.43^{d}	73.33^{b}
Protein (%)	15.23^{cd}	15.00^{d}	17.20^{b}	18.23^{a}	16.50^{c}
Lipids (%)	6.73^{bc}	6.67^{c}	7.17^{ab}	7.37^{a}	7.23^{a}

[1]Mortality rate was 0.0% for all injected fish and the control.
Means having different superscripts within raw are significantly different (P≤0.05).
Initial and final body weight (IBW and FBW) = body weight at start and end of experiment.
Weight gain (WG) = final weight – initial weight.
Daily gain (DG) = (final weight – initial weight) / number of days.
Specific growth rate (SGR%/day) = (Log_e final weight – Log_e initial weight) 100 / number of days.
Condition factor (K) = Body weight (100) / cubic total length.

Table 2. Effect of different doses of shark DNA injected into skeletal muscles of *T. zillii* on biochemical and immunological parameters of blood serum.

Parameters	Doses of shark DNA				
	Control	10 µg	20 µg	40 µg	80 µg
Total protein[1]	5.63^{bc}	5.57^{c}	6.98^{a}	7.25^{a}	5.95^{b}
Albumin[1]	3.20^{a}	3.20^{a}	3.08^{ab}	3.10^{ab}	3.00^{b}
Globulin[1]	2.43^{c}	2.37^{c}	3.90^{a}	4.15^{a}	2.95^{b}
Albumin/Globulin ratio	1.32^{a}	1.36^{a}	0.79^{c}	0.75^{c}	1.02^{b}
IgM [1]	0.77^{c}	0.81^{c}	1.47^{a}	1.55^{a}	0.95^{b}
% IgM [2]	13.68^{c}	14.56^{c}	21.08^{a}	21.38^{a}	15.96^{b}

Means having different superscripts within raw are significantly different (P≤0.05).
[1]g / 100 ml.
[2]Percentage was calculated from total protein.

Our results indicated that the injection of DNA into fish muscle tissues of up to 80µg/fish dose had no lethal effect, as have been previously reported (El-Zaeem, 2004; El-Zaeem and Assem, 2004).

Immunological and biochemical characteristics

The results of immunological and biochemical parameters of blood serum showed a significant effect of different doses of shark DNA injected into *T. zillii*. The highest amounts of serum total protein, globulin, IgM and %IgM were recorded by modified fish injected with 20 and 40 µg/fish. While, the albumin/globulin ratio of the fish injected with 20 and 40 µg/fish was inferior. The lowest significant (P≤0.05) means for serum albumin was recorded by the fish injected with 80µg/fish, but did not differ significantly from that of the fish injected with 20 and 40 µg/fish (Table 2). These results are consistent with our previous work (El-Zaeem and Assem, 2004), where we produced hyperimmune genetically modified Nile tilapia, *O. niloticus* by direct injection of shark DNA into skeletal muscles. Mandour (1996) also observed that a highly immune transgenic chickens can be produced by injecting embryos with various doses of quail bursal (antibody forming cell organ) DNA.

Figure 1. Metaphase chromosomes of diploid (2n=44) *T. zillii.*

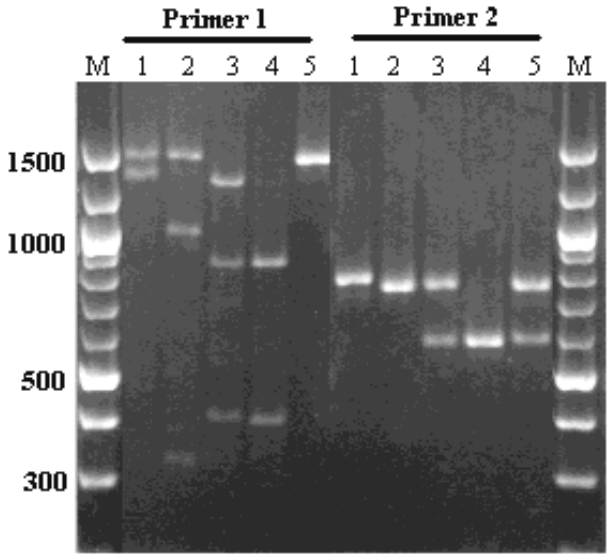

Figure 2. DNA fingerprinting illustrating polymorphism among *T. zillii* muscle tissues following direct injection of various doses of shark DNA. M, 1, 2, 3, 4 and 5 are DNA marker, control, 10µg, 20µg, 40µg and 80µg, respectively.

Cytological examination

In order to investigate the extent effect in other organs, chromosomal complement was studied in kidney tissues. For all injected fish and their control, the cytological examination showed diploid number of *T. zillii* chromosomes (2n=44). Also, no differences in the rate of structural or numerical chromosomal aberrations were detected in all injected fish with different doses of shark DNA and their control (Figure 1). The same findings were reported by El-Fiky and Mehana (1998); El-Zaeem (2004); Hemeida et al. (2004).

Random amplified polymorphic DNA (RAPD) analysis

To evaluate the genetic variability among the modified fish genomes and their control, two random primers were used to determine DNA fingerprinting in muscle tissues injected with various doses of shark DNA as well as control sample. The polymorphic fragments varied between 54.55% for primer 1 and 14.29% for primer 2 (Figure 2). These data showed high polymorphism among normal and modified genomes. It may be due to the differences in the DNA molecule among normal and modified fish as a result of direct injection of different concentrations of shark DNA. Moreover, some fragments of shark DNA may randomly integrated into *T. zillii* muscle genomes. This integration could be functional or silent integration (Yaping et al., 2001). The same observation were reported by El-Zaeem (2001), Ali (2002) and Hemeida et al. (2004) following *in vivo* direct injection of foreign DNA into target fish tissues. Also, the sensitivity of the RAPD marker played an important role in detection of these differences. Baradakci and Skibinski (1994) and Neza et al. (2002) have suggested that the RAPD analysis might be more sensitive than other molecular techniques such mtDNA analysis which has failed to reveal variations within tilapia populations.

Egg diameter and histological characteristics of gonads

The ovaries of control fish had about 15% small ova and 85% yolky ova. The transparent ova counted vary between 3 and 7%, and a number of yolky eggs varied between 9 and 20% as indicated in Figure 3a. Thus the present result revealed that the presence of more ova sizes indicating a long spawning season and the fractional spawning characteristics as indicated by Zaki et al. (1995) for *Oblada melanura* and Assem (2003a) for *Pagellus erythrinus*. The ovary of female *T. zillii* injected with 10 µg/fish of shark DNA had about 28% small ova and 72% yolky ova as indicated in Figure 3b. While the female injected with 20 µg /fish had about 38% small ova and 62% yolky ova as indicated in Figure 3c. The ovary of femal injected with 40 µg /fish had about 43% small ova and 57% yolky ova as indicated in Figure 3d. The ovary of female injected with 80 µg DNA/fish had about 51% small ova and 49% yolky ova as indicated in Figure 3e. In the present results, the number of small ova in ovary of control female was less than that of injected females. Similar observations have been recorded (El-Zaeem and Assem, 2004). Moreover, these results may be related to absence of atretic oocyte in control ovary. The results of the present study support the finding of Clay (1989) and Zaki et al. (1995) for *Oblada melanura*, which indicated that in normal teleost fish the count of small ova was increased at the end of spawning season.

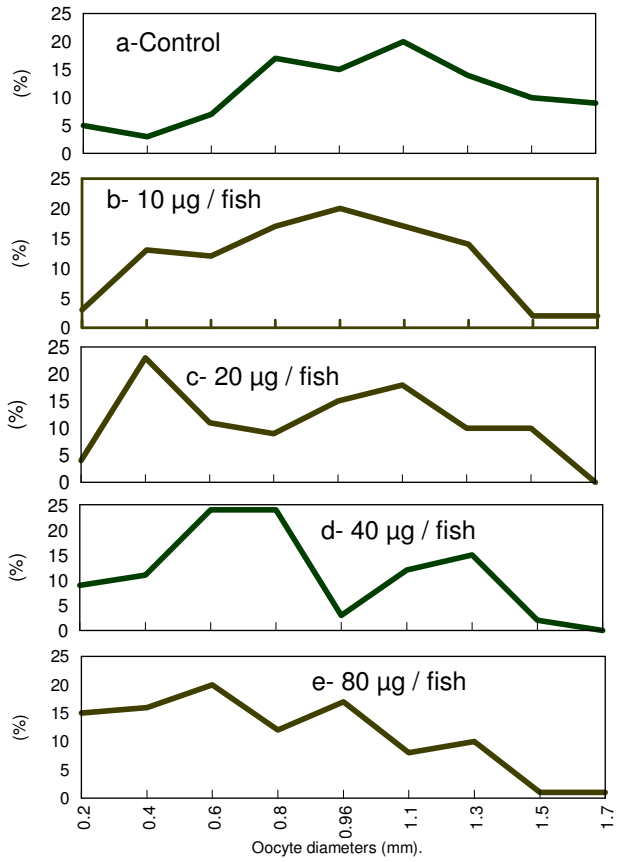

Figure 3. Frequency distribution of *T. zillii* ova diameters for control and injected females.

Thus, the present study indicated that large percentage of small ova diameters was only noticed in the injected fish. This may be related to its quality (atretic or infertile ova) as indicated by Tyler and Sumpter (1996) who pointed that atresia may reduce fecundity for a given fish and also influence egg quality.

For histological characteristics of gonads, ovaries of control female of *T. zillii* were normal in shape and consisted of normal yolk deposition oocytes. The oocytes were rounded or elongated, varied in diameter between 800-1150 µm with a nucleus ranging in diameter from 80-100 µm. The nucleoli were arranged at the periphery of the nucleus; varied in number from 15 to 18 and ranged in diameter from 3-6 µm. The yolk granules were scattered in the cytoplasm and varied in diameter from 8-32 µm. The oocyte wall consisted of zona radiata of about 4 µm in thickness, then granulose layer of about 12 µm in thickness and coated with follicular epithelial layer of about 3 µm in thickness as indicated in Figures 4a. In present study, the wall of oocyte consisted of three layers as indicated by Coward and Bromage (1998) for *T. zillii* and Levavi-Sivan et al. (2004) for Silver perch. Atretic oocytes were found generally occupying less than 2% of the cross section in ovary. In the present study, granulose layer consisted of cuboidal cells in agreement with finding of Coward and Bromage 1998 for *T. zillii*. Coward and Bromage (1998) stated that atresia was not found in pre-vitellogenic developmental stages of *T. zillii*.

The ripe testes of control male of *T. zillii* were composed mainly of nestes or lobules separated by interlobular tissue and showed more active spermatogenesis at all stages of development as shown in Figure 4b. In present study, spermatozoa fill the lobular lumen. Similar observations were reported in catfish Parasilurus aristototelis by Iiadou and Fishelson (1995) and in Pagellus erythrinus by Assem (2003b). The interlobular connective tissue is relatively thin (6 µm in thickness) and rich in interstitial cells and blood capillaries. The observation of histological cross section in *T. zillii*, showed marked asynchronous development in the spermatogenesis during annual reproductive cycle.

In a cross section of the ovary of female *T. zillii* injected with 10 µg/fish, the percentage of normal oocytes was about 10% of the total oocytes. The abnormal oocytes were characterized by absence of follicular epithelium layer, with increase in the granulose layer to reach 40 µm. The zona radiata was swells and loses its striation in places. The yolk granules were coaleces to form large granules reached to 30 µm in diameters as indicated in Figure 5a. In present study there are about 90% of the oocytes in the ovary have abnormal structure as indicated below. Absence of follicular layer with hypertrophy of granulose layer was considered a sign of infertility of the fish due to absence of steroid hormones which used for reproduction as indicated by Yaron et al. (1983) that testosterone and estradiol was produced by follicular and granulose layers of both *O. aureus* and *O. niloticus*.

Cross section in the testes of male *T. zillii* injected with 10 µg/fish, showed all stage of spermatogenesis. The testes were characterized by absence of interlobular connective tissue and a few numbers of spermatozoa. In testes of this fish group, there were vacuoles and spaces free from spermatogenesis as indicated in Figure 5b. The absence of interlobular connective tissue was considered a sign of infertility and shortage in secretion of gonadal hormones as indicated by Cochran (1992) who postulated that the primary function of interlobular connective tissue is to produce steroid needed for gametogenesis and expression of secondary sex characteristics.

The percentage of normal oocytes in ovary of female *T. zillii* injected with 20 µg/fish was about 38% from total oocytes. The abnormal oocytes were characterized by absence of follicular epithelium layer. In some places the follicular layer becomes deflected from its original position. Zona radiata was thrown into folds, and in some places it was deflected from its original position and penetrate the oocyte content. The yolk granules coalesce to form irregular masses. Residues of atretic oocytes embedded between the normal oocytes as indicated in Figure 6a. In the present study, the abnormal wall of the

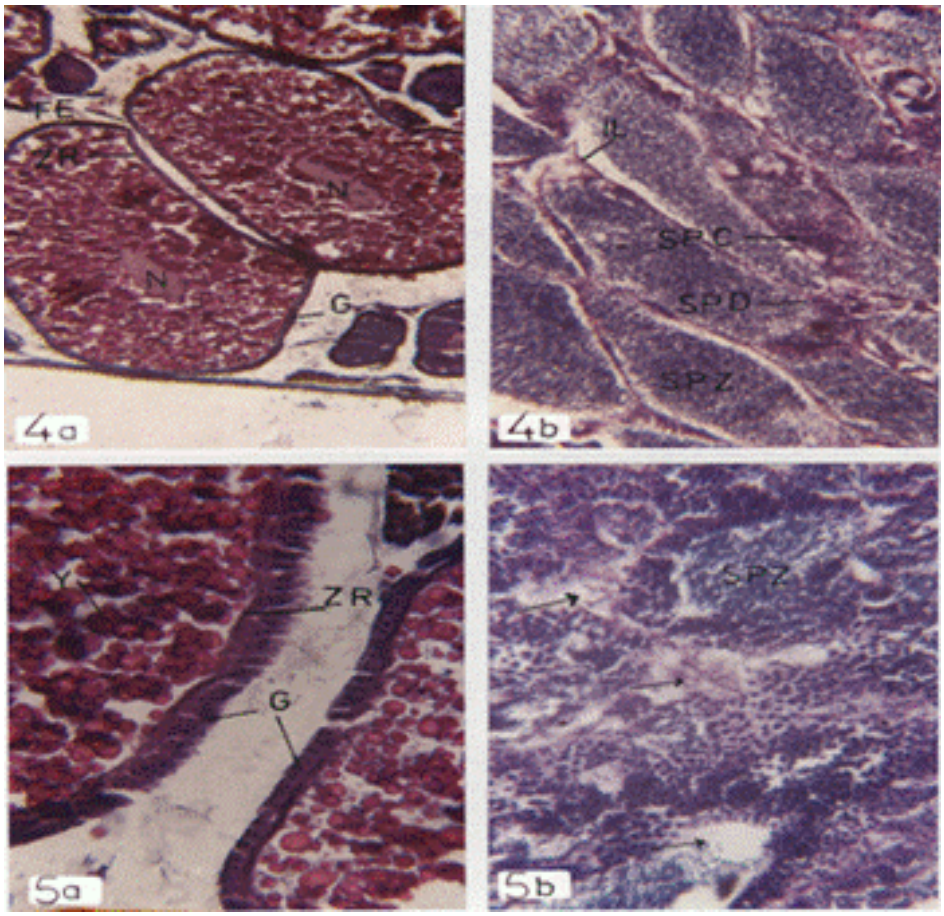

Figure 4a. Photomicrograph of cross section (CS) in control ovary of *T. zillii* showing, zona radiata (ZR), granulose layer (G), follicular epithelial layer (FE) and nuclei (N). Stained (H&E) X100. **4b.** Photomicrograph of CS in control testes of *T. zillii* showing, different stages of spermatogenesis, secondary spermatocytes (SPC), spermatids (SPD), spermatozoa (SPZ) and interlobular connective tissue (IL). (H & E). X250.

Figure 5a. Photomicrograph of CS in ovary of *T. zillii* injected with 10µg /fish showing, absence of follicular epithelium layer, increase in thickness of granulose layer (G) & zona radiata (ZR) and the yolk globules (Y) coalesce to form large globules. (H&E) X 100. **5b.** Photomicrograph of CS in testes of *T. zillii* injected with 10 µg/fish of shark DNA showing, absence of interlobular connective tissue, few number of spermatozoa (SPZ), appearance of spaces free from cells (arrows), (H&E) X250

oocytes indicate that the oocyte is atretic and will be phagocytic as explained by Ramadan et al. (1987) indicated that deformation of the wall in the oocyte was considered as the first step of atresia and phagocytosis of the oocyte in situe.

Testes in the cross section of male injected with 20 µg/fish were characterized by deflection of interlobular tissue from its original position, in some places the interlobular tissue were absent. The lobules were surrounded with vacuoles and degenerated fibers. Many lobules were free from spermatozoa as indicated in Figure 6b. In present study, the abnormal shape of interlobular connective tissue was considered as an evidence of infertility and shortage in secretion of steroid hormone as indicated by Sulistyo et al. (2000).

In cross section in ovary of female *T. zillii* injected with 40 µg/fish, the percentage of normal oocytes was about 65% of the total number of oocytes. The abnormal oocytes were characterized by shrinks of follicular epithelial layer; in some places the follicular layer was absent. There were hypertrophies of granulose layer, with spaces between granulose cells. Zona radiata was

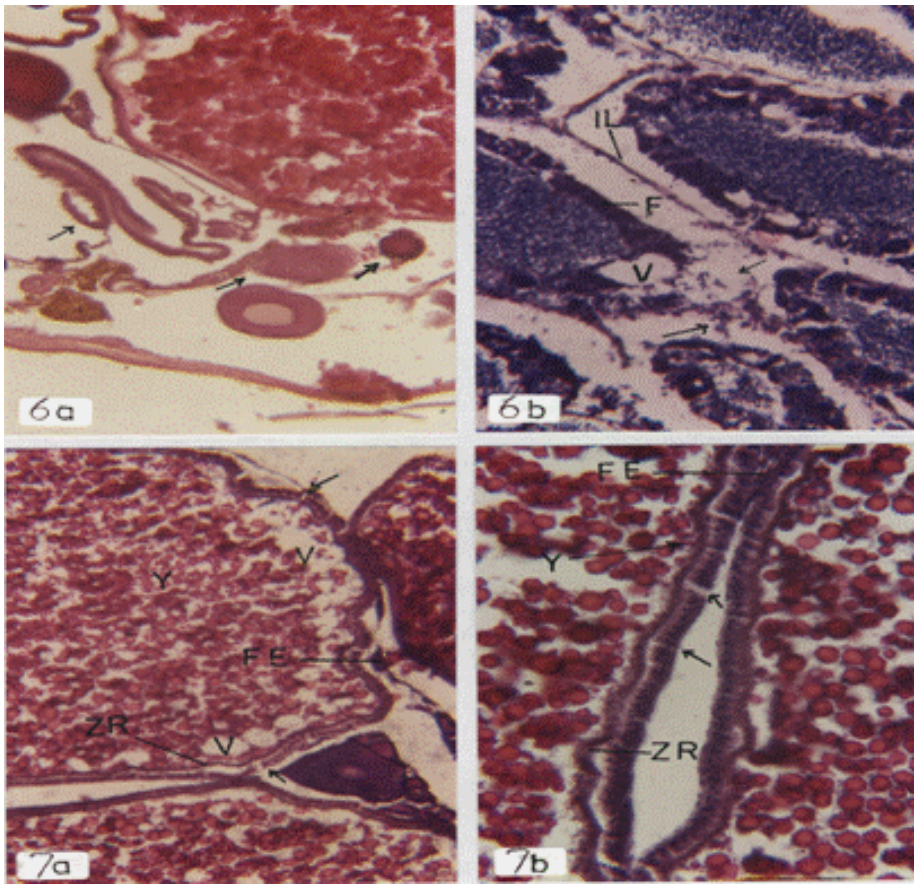

Figure 6a. Photomicrograph of CS in ovary of *T. zillii* injected with 20 µg /fish showing, residues of atretic oocytes (arrows) embeded between normal oocytes. (H &E) X 100. **6b.** Photomicrograph of CS in testes of *T. zillii* injected with 20 µg/fish showing, deflection of interlobular connective tissue (IL), in some places absent (arrows). The lobules was filled with vacuoles (V) and degenerated fibers (F), (H&E) X 250.

Figure 7a,b. Photomicrograph of CS in ovary of *T. zillii* injected with 40 µg/fish showing, shrinks of follicular epithelium (FE), and spaces between granulose cells (arrows), large vacuoles in the peripheral of the oocyte (V). Yolk granules broken into small fragment (Y), irregular zona radiata (ZR) (H &E) X 100 and X250 respectively.

irregular in shape and in some places was deflected from its original position. There were large vacuoles surround the oocyte content. The yolk granules were broken into small fragments, in some places the yolk granules coalesce to form irregular masses as indicated in Figures 7a,b. In the present results, abnormal shape of follicular epithelial layer with absent of its special theca cells considered as a sign of infertility and shortage of hormonal secretion as indicated by Nagahama et al. (1995) who concluded that testosterone is produced by special theca cells. Steroidogenesis is much more complicated in asynchronous ovaries as in tilapia. Planas et al. (2000) recorded that a significant increase in basal reproduction of 17, 20-β-dihydroxy-4-pregnen-3-one by theca and granulose layer was recorded in salmonid fish.

In cross section of the testes of male injected with 40 µg /fish of shark DNA, spermatozoa were detected with few number of spermatids. The sections were characterized by presence of vacuoles and spaces free from spermatogenesis as indicated in Figure 7c. These testes were considered as more similar to normal tilapia, the percentage of similarity about 70%, the interlobular connective tissue was normal in shape.

The percentage of normal oocytes of the ovary of female injected with 80 µg /fish of shark DNA was about 18%. The abnormal oocytes were characterized by hypertrophy of follicular epithelial and granulose layers. The granulose layer consisted of many rows of cells and measured about 96 µm. Zona radiata was thrown into folds and in some places was deflected from its original position and penetrate the oocyte content. There were fragmentation in yolk granules to form minute granules measured 3 µm in diameters as indicated in Figure 8a. Hypertrophy and convoluted of granulose layer

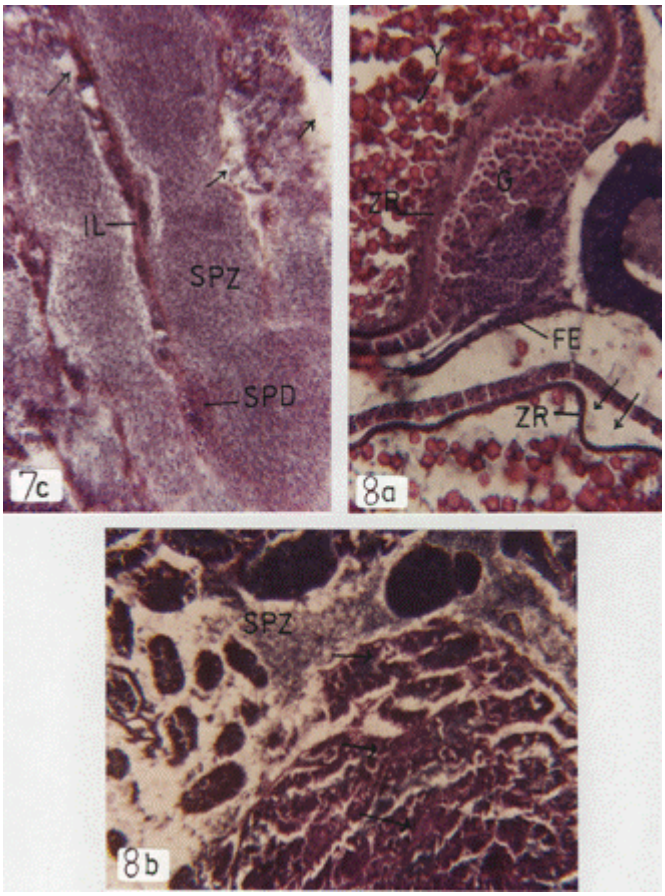

Figure 7c. Photomicrograph of CS in testes of *T. zillii* injected with 40 µg/fish showing, spermatozoa (SPZ), spermatids (SPD), undifferentiated fibers surrounde the interlobular connective tissue (IL), spaces free from spermatogenesis (arrows), (H&E) X 250.

Figure 8a. Photomicrograph of CS in ovary of *T. zillii* injected with 80 µg /fish showing, hypertrophy of follicular epithelial layer (FE), the granulose layer consisted of many rows (G). Fragmentation of yolk granules (Y). Zona radiata was thrown into folds (ZR) and penetrate the oocyte content with free spaces (arrows). (H &E) X 250. **8b.** Photomicrograph of CS in testes of *T. zillii* injected with 80 µg/fish showing, atretic lobules filled with many vacuoles and undifferentiated tissue (arrows) and few number of spermatozoa (SPZ), (H&E) X 250.

considered first step in atresia of the oocyte as indicated by Casadevall et al. (1993); the atretic body is considered to be an ovarian follicle, containing a developing oocyte, which has degenerated spontaneously. The oocyte does not detach itself, but rather it involutes and a fibrous or granular structure of doubtful interpretation appears.

The testes of male injected with 80 µg/fish of shark DNA were characterized by absence of interlobular connective tissue. In section of testes there were vacuoles and spaces free from spermatogenesis. The spermatozoa were few in numbers intermingled with undifferentiated tissue as indicated in Figure 8b.

The comparison between all injected fish revealed that the testes and ovaries of the fish injected with 80 µg/fish were characterized by deformed and atretic gonads more than the fish in other concentration. These results are consistent with the findings reported by El-Zaeem and Assem (2004).

Penman et al. (1990) reported that there were a trend towards increased mortality of rainbow trout eggs with increasing DNA concentration. The present results are consistent with this finding and strongly support the hypothesis that the highest doses of foreign DNA lead to a delay in spawning time and decrease of the productive performance of fish (El-Zaeem, 2001, 2004; El-Zaeem and Assem, 2004). This may be due to destruction of the transgene copies by nuclease activity as the result of highest doses of foreign DNA (Rahman and Maclean, 1992).

Sudha et al. (2001) reported that the expression of intramuscular injection of foreign DNA is evident in several nonmuscle tissues of fish, such as skin, epithelia, pigment cells, blood vessel cells and neuron-like cells. The present results are consistent with these findings, whereas the effects of intramuscular injection of shark DNA into *T. zillii* could be limited to blood and germ cells. Thus, further studies about the establishment of these effects on the following generations are needed. Finally, the results indicate that, hyperimmune genetically modified *T. zillii* with accelerated growth can be produced with a feasible and fast methodology.

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